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## Increased dietary NaCl potentiates the effects of elevated prorenin levels on blood pressure and organ pathology

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### Abstract

**Background**—Rats with several hundred-fold elevation of plasma prorenin levels due to liver-specific expression of a rat prorenin transgene have cardiac and aortic hypertrophy, renal lesions and myocardial fibrosis. The effect of increased dietary NaCl on the phenotype of prorenin transgenic rats has not been examined.

**Methods and results**—We compared the effects of 0.3% and 2% dietary NaCl in wildtype and transgenic rats from 3–12 months of age. In comparison with wildtype rats, transgenic rats receiving 0.3% dietary NaCl had ~1000-fold elevation of prorenin, 1.5- to 2.5-fold elevation of renin concentration and activity, wildtype levels of angiotensin II, and were hypertensive with cardiac and aortic hypertrophy, and increased renal glomerular and tubulo-interstitial injury score. In wildtype rats, 2% dietary NaCl reduced angiotensin levels, produced a delayed increase in blood pressure, and caused cardiac hypertrophy and tubulo-interstitial injury. By contrast, 2% NaCl did not reduce angiotensin levels in transgenic rats, potentiated their hypertension, cardiac and aortic hypertrophy, and increased myocardial interstitial and perivascular fibrosis, without effect on glomerular or tubulo-interstitial injury score.

**Conclusion**—Increased dietary NaCl had a greater impact on the phenotype of transgenic than wildtype rats that may have been due in part to their hypertension and their failure to suppress angiotensin levels, consequent to their elevated prorenin levels.

### Keywords

prorenin; transgene; dietary NaCl; hypertension; angiotensin II; nephrosclerosis; cardiac hypertrophy; cardiac fibrosis

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## Introduction

Prorenin, the biosynthetic precursor of renin, contains a prosegment that masks the active site, thereby preventing access by the renin substrate, angiotensinogen [1,2]. Plasma prorenin concentrations are 10- to 20-fold higher than renin concentrations in humans [3,4], and there is controversy whether plasma prorenin has biological activity *in vivo* [1,2]. Partial conversion to renin, and a low degree of intrinsic activity due to transitory unfolding of the prosegment, may contribute to angiotensin formation by prorenin. In addition, prorenin binding to the (pro)renin receptor may initiate signal transduction by mechanisms independent of angiotensin peptide formation, and the (pro)renin receptor may activate prorenin by promoting unfolding of the prosegment [1,2,5].

Transgenic animal models with elevated prorenin levels are valuable for study of the role of prorenin *in vivo*. We previously reported the (hAT-rpR) rat with liver-specific expression of a rat prorenin transgene under the control of a human  $\alpha$ 1-antitrypsin promoter. Plasma prorenin levels of male (hAT-rpR) rats were ~600-fold higher than in wildtype (WT) rats, and male (hAT-rpR) rats exhibited cardiac hypertrophy and severe renal lesions, hypertrophic cardiomyocytes, interstitial and perivascular fibrosis in the heart, increased aortic wall thickness, and suppressed kidney renin levels, despite WT levels of plasma renin activity (PRA) and systolic blood pressure (SBP) [6]. However, in contrast to our initial report of male (hAT-rpR) rats housed in Edinburgh [6], we subsequently found that male (hAT-rpR) rats housed in Paris were hypertensive by 3 months of age, and developed modest renal lesions and cardiac fibrosis only after 6 months of age [7]. Plasma and kidney angiotensin (Ang) II levels of male (hAT-rpR) rats were similar to the levels in WT littermates, and nephrectomy experiments showed liver-derived prorenin was the major contributor to plasma Ang II levels in (hAT-rpR) rats that likely contributed to their hypertension, cardiac hypertrophy and suppressed kidney renin levels.

We were unable to explain the differences in phenotype of (hAT-rpR) rats in our two studies. Given the hypertension and more benign phenotype of (hAT-rpR) rats housed in Paris, we were interested to explore factors that may modify their phenotype. Increased dietary NaCl is well recognized to cause hypertension and cardiovascular disease and to amplify the hypertensive and pathogenic effects of Ang II [8-10], and the mouse prorenin transgenic (mRen-2)27 rat is particularly susceptible to dietary NaCl [11]. We therefore studied the effect of 2% NaCl, in comparison with 0.3% NaCl, from 3 to 12 months of age on blood pressure, the renin angiotensin system, and organ pathology in male WT and (hAT-rpR) rats. We hypothesized that (hAT-rpR) rats may not suppress Ang II levels and may therefore show an exaggerated increase in blood pressure and organ pathology in response to increased dietary NaCl.

## Materials and Methods

The generation of (hAT-rpR) rats by the Centre for Genome Research, Edinburgh, has been described [6]. This transgenic rat line was created by inserting a rat prorenin cDNA fused to a human  $\alpha$ 1-antitrypsin promoter into the genome of the Fischer F344 rat. In December 1996, 9 male and 12 female heterozygous (hAT-rpR) rats, strain 85-26, and 8 WT male Fischer F344 rats were received at INSERM U367, Paris, France. The colony was maintained by mating heterozygous male and female (hAT-rpR) rats. Because expression of the rat prorenin transgene in (hAT-rpR) rats showed sexual dimorphism and because only male rats showed renal, cardiac and aortic abnormalities [6], our study was confined to heterozygous male (hAT-rpR) rats and their WT male littermates, and was carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All rats were genotyped by PCR of DNA

obtained from tail biopsies as previously described [7]. The rat experiments were completed in 1999.

### Experimental procedures

Rats were housed in groups of 4-5 animals, lights on from 08.00-19.00, and were fed a normal rat diet containing 0.3% NaCl, and tap water *ad libitum*. At 3 months of age, WT and (hAT-rpR) rats were randomized to either continue the normal rat diet or to commence an equivalent diet with 2% NaCl. Both diets contained 0.9% potassium. We previously showed these diets, prepared by UAR, Epinay-sur-Orge, France, ensure similar rat growth rates [12]. Food intake was not measured. SBP and body weight were measured every 3 months; SBP was measured in conscious rats using tail-cuff plethysmography.

### Collection of blood and tissues

At 3 months of age (just before commencement of 2% NaCl diet) and at 6, 9, and 12 months of age, blood (1 mL) for measurement of PRA, plasma renin concentration (PRC), prorenin and angiotensinogen was taken from the jugular vein under light anaesthesia with ketamine-xylozine (15 and 5 mg/kg body weight, respectively, by intra-peritoneal injection) [12] into a syringe with ~0.05 vol 2.5 mmol/L  $\Omega$ -phenanthroline, 0.1 mol/L EDTA to inhibit angiotensin converting enzyme and angiotensinase activity, and centrifuged at 4,000 *g* for 10 min. Plasma was stored at -80°C for subsequent assay. At 12 months of age, after collection of blood from the jugular vein, blood (10 mL) for measurement of angiotensin peptides was collected from the aorta into a syringe containing 0.5 mL 0.1 mmol/L rat renin inhibitor kindly provided by Dr Hiwada [13], 0.01 mmol/L MK 422 and 1 mmol/L EDTA. The organs were sampled immediately after blood collection. A portion of liver was frozen in liquid nitrogen for RNA extraction. The left and right ventricles were dissected, blotted and weighed. The kidneys were cut longitudinally into 2 equal portions and one portion was frozen in liquid nitrogen for renin measurement. The other portion of kidney was placed in alcoholic Bouin's fixative and the heart and the abdominal aorta were placed in 4% paraformaldehyde for histological and morphometric analyses.

### Measurement of plasma prorenin, renin, angiotensin peptides and angiotensinogen, and renal renin

Plasma prorenin, PRA, PRC, angiotensin peptides and angiotensinogen, and renal renin were measured as previously described [7].

### Northern blot analysis

Total RNA was extracted from liver using Tri-Reagent solution (Molecular Research Centre), electrophoresed on 1% agarose gels, transferred to supercharged nylon membrane (Schleicher & Schuell) and fixed by UV cross-linking. The Northern blots were probed for renin mRNA using <sup>32</sup>P-labeled mouse submaxillary gland renin cDNA prepared using a random primer labeling kit (Prime-It II, Stratagene). Renin hybridization signal was normalized to that of GAPDH.

### Analysis of renal histology

Kidneys from male (hAT-rpR) and WT rats, fixed in alcoholic Bouin's solution, were embedded in paraffin, and 2 sagittal sections (4  $\mu$ m) were stained with Masson's trichrome and examined under light microscopy. Two investigators blinded as to the experimental groups graded the severity of the morphological changes semiquantitatively as described previously [7,14,15]. For assessing glomerulosclerosis, 30 superficial and 30 juxtamedullary glomeruli in each kidney were observed at X400 magnification and a glomerular damage index was calculated for the 60 glomeruli [7]. For tubulo-interstitial lesions (interstitial

inflammation and fibrosis, tubular atrophy and casts) every third field of each kidney (total of 10 fields/kidney) was assessed at X100 magnification and the score corresponding to tubulo-interstitial lesions was obtained for each kidney [7]. The vascular lesion score was obtained as described previously [7].

### Analysis of cardiac fibrosis and aortic medial hypertrophy

Heart and abdominal aorta from male (hAT-rpR) and WT rats, fixed in 4% paraformaldehyde, were embedded in paraffin. Two coronal ventricular sections (4  $\mu\text{m}$ ) were taken at the equator of the heart and 3 sequential sections (4  $\mu\text{m}$ ) were taken from the mid portion of the abdominal aorta. The cardiac sections were stained for collagen with picrosirius red and the aortic sections were stained for elastin with orcein. Cardiac and aortic measurements were determined by quantitative morphometry of digitized images using Aperio instrumentation (Aperio Technologies, Inc., CA). Left ventricular myocardial interstitial collagen density was calculated using the positive pixel count algorithm as the area of collagen staining expressed as a percentage of the total myocardial tissue area, after excluding perivascular fibrosis. Perivascular fibrosis and wall/lumen ratio of intramyocardial vessels were measured by planimetry of vessels with a maximum/minimum cross-sectional diameter ratio  $<1.5$  and average maximum diameter  $\sim 60 \mu\text{m}$  (range 20-130  $\mu\text{m}$ ), and the median value of an average of 4-6 vessels was determined for each rat. Perivascular fibrosis was calculated as the ratio of the area of perivascular fibrosis to the total vessel area (area of vessel wall plus lumen) [16]. The wall/lumen ratio was calculated as the ratio of the area of the vessel wall to the lumen area.

Abdominal aortic cross-sectional area, wall area, and outer and inner aortic wall circumference were estimated by planimetry. Two sections were analyzed for each rat. The aortic wall thickness was calculated as the ratio of the wall area to the average mid-circumference of the aortic wall. Investigators responsible for morphometric analyses were blinded as to experimental group.

### Statistical analysis

Data are expressed as means $\pm$ SEM. Calculations were done with Statview 5.0.1 statistical software (SAS Institute Inc.), and a value of  $P<0.05$  was considered significant. Body weight, SBP, prorenin, PRC and PRA were analyzed by repeated measures analysis of variance (ANOVA). Other data were analyzed by 2-way ANOVA. Data were logarithmically transformed when necessary to normalize variances. The Fisher's Protected Least Significant Difference test was used for multiple comparisons.

## Results

### Body weight, SBP, and left ventricular and kidney weights

The effects of 2% dietary NaCl on body weight and systolic blood pressure are shown in Figure 1. There was no difference in body weight between WT and (hAT-rpR) rats, and 2% dietary NaCl did not influence body weight of either genotype, when analyzed by 2-way repeated measures ANOVA for 3-12 months of age. However, separate analysis of 12 month body weight data showed the body weight of (hAT-rpR) rats receiving 2% dietary NaCl was less than that of WT rats receiving 2% dietary NaCl (Figure 1). Blood pressure was higher in (hAT-rpR) than in WT rats, and 2% dietary NaCl increased blood pressure of both genotypes. The increase in blood pressure with 2% dietary NaCl was more rapid for (hAT-rpR) than for WT rats, but by 12 months of age, after 9 months of 2% dietary NaCl, the absolute blood pressure increase was similar for the 2 genotypes. At 12 months of age, SBP was  $148\pm 5$  mmHg (mean  $\pm$  SEM,  $n = 7$ ) and  $170\pm 4$  mmHg ( $n = 8$ ) for WT and (hAT-rpR)

rats, respectively, receiving 0.3% NaCl, and 2% dietary NaCl increased SBP of WT rats to  $164 \pm 7$  mmHg ( $n = 8$ ) and SBP of (hAT-rpR) rats to  $187 \pm 7$  mmHg ( $n = 8$ ).

Left ventricular weight and left ventricular weight/body weight ratio were higher in (hAT-rpR) than WT rats, and 2% dietary NaCl increased left ventricular weight and left ventricular weight/body weight ratio for both genotypes (Table 1). There were no differences between WT and (hAT-rpR) rats for kidney weight or kidney weight/body weight ratio; 2-way ANOVA showed a significant increase in kidney weight/body weight ratio with 2% dietary NaCl, but this effect of 2% dietary NaCl was not significant when tested for each genotype separately (Table 1).

### Plasma prorenin, renin, angiotensin peptides and angiotensinogen, and renal renin

Plasma prorenin, PRC, and PRA were measured at 3, 6, 9, and 12 months of age (Figure 2). At 12 months of age, prorenin levels were  $68979 \pm 22788$  (mean  $\pm$  SEM,  $n = 8$ ) ng Ang I/mL per h and  $16938 \pm 3981$  ( $n = 8$ ) ng Ang I/mL per h for 0.3% and 2% dietary NaCl, respectively in (hAT-rpR) rats and  $69 \pm 15$  ( $n = 7$ ) ng Ang I/mL per h and  $30 \pm 10$  ( $n = 7$ ) ng Ang I/mL per h for WT rats. Thus, prorenin levels of (hAT-rpR) rats were ~1000-fold higher than in WT rats and, in comparison with 0.3% dietary NaCl, 2% dietary NaCl reduced prorenin by ~75% in (hAT-rpR) rats and by ~56% in WT rats. PRA and PRC of (hAT-rpR) rats were 1.5- to 2.5-fold higher than in WT rats and, in comparison with 0.3% dietary NaCl, 2% dietary NaCl reduced PRA by ~39% in (hAT-rpR) rats and by ~49% in WT rats, but did not reduce PRC in either genotype at 12 months of age.

Plasma Ang I and Ang II, angiotensinogen and kidney renin were measured at 12 months of age (Table 1). Ang II levels of (hAT-rpR) rats were similar to WT levels but Ang I levels were lower than WT levels on 0.3% dietary NaCl. Increased dietary NaCl reduced Ang I and Ang II levels in WT, but not in (hAT-rpR), rats. Plasma angiotensinogen levels were lower in (hAT-rpR) than in WT rats, and were not influenced by 2% dietary NaCl. Kidney renin levels were suppressed in (hAT-rpR) rats; whereas 2% dietary NaCl reduced kidney renin levels in WT rats, it had no effect on the already suppressed kidney renin levels in (hAT-rpR) rats.

### Liver renin mRNA levels

In agreement with the reduction in plasma prorenin levels by 2% dietary NaCl, Northern blot analysis at 12 months of age showed a 37% reduction in hepatic renin mRNA levels in (hAT-rpR) rats; renin/GAPDH ratio:  $11.4 \pm 0.7$  (mean  $\pm$  SEM,  $n=4$ ) for 0.3% dietary NaCl,  $7.2 \pm 1.2$  ( $n=5$ ) for 2% dietary NaCl,  $P = 0.02$ . Renin mRNA was not detectable in livers of WT rats.

### Renal histology

At 12 months of age the proportion of normal glomeruli for WT rats was 98% (range 95-100%) for 0.3% dietary NaCl and 98% (93-100%) for 2% dietary NaCl, and for (hAT-rpR) rats was 90% (73-100%) for 0.3% dietary NaCl and 89% (73-100%) for 2% dietary NaCl. Glomerular injury score was higher in (hAT-rpR) than in WT rats but 2% dietary NaCl did not affect glomerular injury score in either genotype (Figures 3 and 4).

The proportion of normal tubulo-interstitium for WT rats was 91% (range 70-100%) for 0.3% dietary NaCl and 60% (30-90%) for 2% dietary NaCl, and for (hAT-rpR) rats was 41% (10-90%) for 0.3% dietary NaCl and 28% (0-70%) for 2% dietary NaCl. Tubulo-interstitial injury score was higher in (hAT-rpR) than in WT rats and 2% dietary NaCl increased tubulo-interstitial injury score in WT, but not in (hAT-rpR), rats. None of the WT rats receiving 0.3% or 2% dietary NaCl and none of the (hAT-rpR) rats receiving 0.3%



dietary NaCl had vascular lesions; only 2 of the 8 (hAT-rpR) rats receiving 2% dietary NaCl had vascular lesions. Thus, neither genotype nor dietary NaCl affected kidney vascular injury score.

### Aorta

Abdominal aortic cross-sectional area, wall area and thickness were higher in (hAT-rpR) than in WT rats (Table 1). While without effect on aortic wall area and thickness in WT rats, 2% dietary NaCl increased both parameters in (hAT-rpR) rats. However, dietary NaCl did not influence aortic cross-sectional area of either genotype.

### Cardiac histology

Myocardial interstitial and perivascular fibrosis and myocardial vessel wall/lumen ratio were similar for (hAT-rpR) and WT rats receiving 0.3% dietary NaCl (Figures 5 and 6). Increased dietary NaCl increased myocardial interstitial and perivascular fibrosis in (hAT-rpR), but not in WT, rats; however, dietary NaCl did not influence myocardial vessel wall/lumen ratio of either genotype.

### Discussion

We found that an increase in dietary NaCl from 0.3% to 2% produced a greater impact on the phenotype of (hAT-rpR) than of WT rats. Increased dietary NaCl potentiated the hypertension, cardiac and aortic hypertrophy, and increased myocardial interstitial and perivascular fibrosis, without effect on myocardial vessel wall/lumen ratio, or renal glomerular, tubulo-interstitial, or vascular injury score in (hAT-rpR) rats. By contrast, 2% dietary NaCl produced only a delayed increase in blood pressure of WT rats, in association with an increase in left ventricular weight and tubulo-interstitial injury, but no other cardiac, renal, or vascular consequences. The greater susceptibility of (hAT-rpR) rats to increased dietary NaCl may have been due in part to their hypertension and their failure to suppress angiotensin levels in response to increased dietary NaCl. As we have previously reported for (hAT-rpR) rats housed in Paris, ~1000-fold increase in prorenin levels had a modest impact on cardiac, renal and vascular morphology, in association with increased blood pressure [7]. Increased prorenin levels did not affect myocardial interstitial or perivascular fibrosis in the present study, although 2% dietary NaCl increased myocardial interstitial and perivascular fibrosis in (hAT-rpR), but not in WT, rats.

Increased dietary NaCl produced the expected suppression of plasma prorenin, PRC, PRA and angiotensin peptide levels, and kidney renin levels in WT rats. The suppression of plasma prorenin and PRA in (hAT-rpR) rats was, however, unexpected. The reduction in prorenin mRNA levels in liver of (hAT-rpR) rats suggested an effect of 2% dietary NaCl on transcription of the transgene, which was under the control of the human  $\alpha$ 1-antitrypsin promoter. However, rat hepatic  $\alpha$ 1-antitrypsin mRNA levels are unresponsive to 8% dietary NaCl [17], and it is therefore unknown why the human  $\alpha$ 1-antitrypsin promoter of the rat prorenin transgene may have responded to increased dietary NaCl. The markedly lower kidney renin levels in (hAT-rpR) than WT rats on 0.3% NaCl diet was confirmation of our earlier studies [6,7], and the failure of 2% dietary NaCl diet to further reduce kidney renin levels in (hAT-rpR) rats was consistent with these levels representing trapped plasma prorenin in the tissue, that was subsequently activated during tissue processing before renin assay.

Although (hAT-rpR) rats had higher PRC and PRA levels than WT rats on 0.3% dietary NaCl, their angiotensin peptide levels were not elevated. As we have previously discussed [7], the higher PRC and PRA levels in (hAT-rpR) rats were probably due to in part to

inadvertent *in vitro* activation of the ~1000-fold elevated prorenin levels during collection and processing of blood and performance of the PRC and PRA assays. Thus, the fall in PRA in (hAT-rpR) rats given 2% dietary NaCl was likely a consequence of the fall in prorenin levels. However, given that our previous studies of the effects of nephrectomy indicated a predominant contribution of liver-derived prorenin to angiotensin peptide formation in (hAT-rpR) rats, we are unable to explain why the lower prorenin levels in (hAT-rpR) rats given 2% dietary NaCl were not accompanied by lower Ang I and Ang II levels, although these data raise the possibility that dietary NaCl may modulate the contribution of prorenin to angiotensin peptide formation.

Most previous studies of the effects of increased dietary NaCl in rats used 8% dietary NaCl, which increased blood pressure and induced cardiac and renal hypertrophy and myocardial and renal fibrosis in both normotensive and spontaneously hypertensive rats [8,9]. Our comparison of 0.3% and 2% dietary NaCl was more physiological in that the 7-fold difference in NaCl intake was less than the differences in NaCl intake used in some clinical studies [18,19]. Simon and Illyes found no effect of 2% dietary NaCl on blood pressure of Sprague Dawley rats over 12 weeks [10], in agreement with the present finding that greater than 3 months duration of 2% dietary NaCl was required to increase blood pressure of WT rats. Increased dietary NaCl is well recognized to amplify the hypertensive and pathogenic effects of Ang II [8-10]. Ang II administration at a dose that was suppressor in rats given 0.7% dietary NaCl was pressor and produced vascular hypertrophy in rats given 2% dietary NaCl [10]. Thus, the lack of suppression of Ang II levels by 2% dietary NaCl in (hAT-rpR) rats would be predicted to exacerbate the effects of increased dietary NaCl in these rats. The impact of 2% dietary NaCl on the phenotype of (hAT-rpR) rats was less than that reported for mouse prorenin transgenic (mRen-2)27 rats. In comparison with 0.45% dietary NaCl, male heterozygous (mRen-2)27 rats fed 2% NaCl from the age of 1 to 6 months developed a rapid increase in blood pressure with transition to malignant phase hypertension, together with increased cardiac hypertrophy, proteinuria, and glomerulosclerosis [11]. The greater effect of 2% dietary NaCl in (mRen-2)27 rats may have been due to their greater elevation of plasma Ang II levels [20,21].

In summary, we found an increase in dietary NaCl from 0.3% to 2% had a much greater impact on the phenotype of (hAT-rpR) than WT rats. Increased dietary NaCl potentiated the hypertension, cardiac and aortic hypertrophy, and myocardial interstitial and perivascular fibrosis of (hAT-rpR) rats, without modification of myocardial vessel wall/lumen ratio or renal histology. The increased susceptibility of (hAT-rpR) rats to increased dietary NaCl may have been due in part to their hypertension and their failure to suppress angiotensin levels, consequent to their elevated prorenin levels, and to an effect of dietary NaCl on angiotensin peptide formation by prorenin. It is of note, however, that plasma prorenin levels of (hAT-rpR) rats were ~1000-fold higher than physiological levels and further studies are required to define the contribution of prorenin to organ pathology in conditions with lesser degrees of elevation of prorenin levels, such as diabetes and pregnancy.

#### Condensed abstract

Rats with several hundred-fold elevation of plasma prorenin levels due to liver-specific expression of a rat prorenin transgene have cardiac and aortic hypertrophy, renal lesions and myocardial fibrosis. We compared the effects of 0.3% and 2% dietary NaCl in wildtype and transgenic rats from 3-12 months of age. In contrast to wildtype rats, 2% NaCl did not reduce angiotensin levels in transgenic rats, potentiated their hypertension, cardiac and aortic hypertrophy, and increased myocardial interstitial and perivascular



fibrosis. We conclude that increased dietary NaCl had a greater impact on the phenotype of transgenic than wildtype rats.

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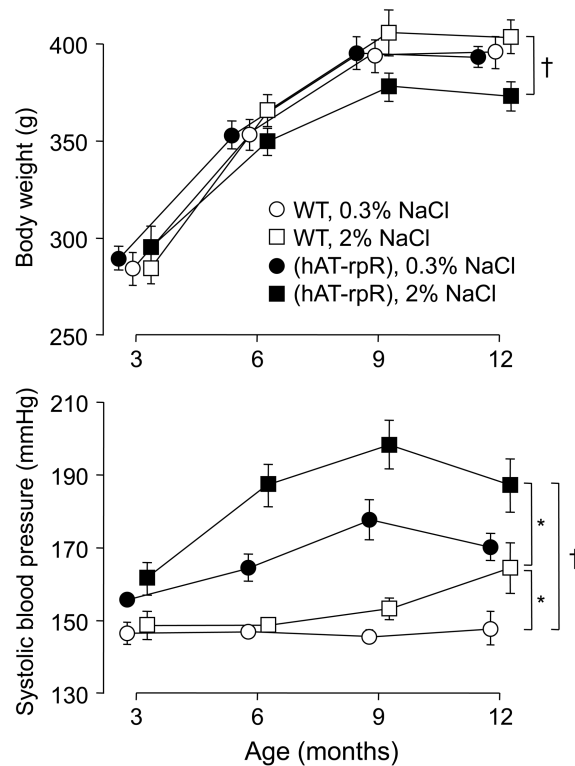
## Abbreviations

<b>(hAT-rpR)</b>	rats with liver-specific expression of a rat prorenin transgene under the control of a human $\alpha 1$ -antitrypsin promoter
<b>(mRen-2)27</b>	rats transgenic for mouse Ren-2 prorenin
<b>Ang</b>	angiotensin
<b>BW</b>	body weight
<b>KW</b>	kidney weight
<b>LVW</b>	left ventricular weight
<b>PRA</b>	plasma renin activity
<b>PRC</b>	plasma renin concentration
<b>SBP</b>	systolic blood pressure
<b>WT</b>	wildtype

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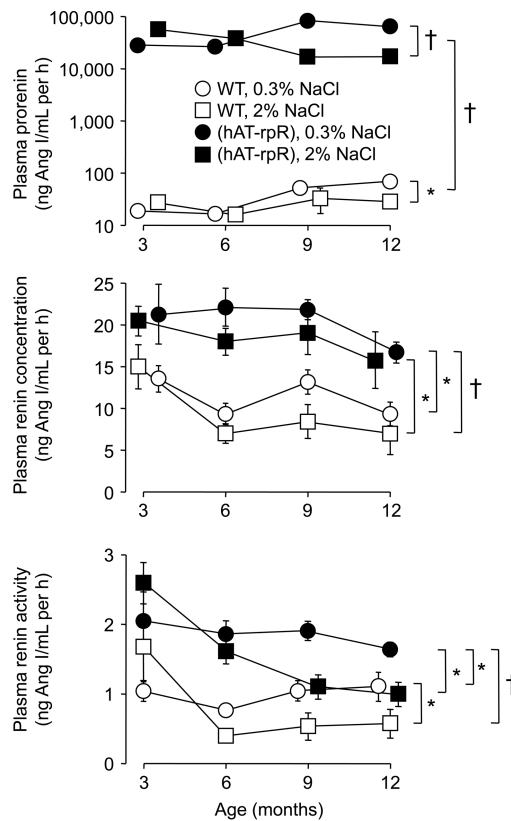
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**Figure 1.**

Effect of 0.3% (circles) and 2% (squares) dietary NaCl on body weight (BW) and systolic blood pressure (SBP) in male wildtype (WT, open symbols) and (hAT-rpR) rats (closed symbols). Data shown as means  $\pm$  SEM,  $n=7-8$ . For BW, 2-way repeated measures ANOVA for 3-12 months of age showed no effect of genotype, dietary NaCl, or interaction between genotype and dietary NaCl. However, when BW at 12 months was analysed separately, there was a significant effect of genotype ( $P=0.03$ ) but not dietary NaCl, and (hAT-rpR) rats receiving 2% dietary NaCl had lower BW than WT rats receiving 2% dietary NaCl ( $P=0.006$ ). For SBP, 2-way ANOVA for 3-12 months of age showed higher SBP in (hAT-rpR) than WT rats ( $P<0.0001$ ), and in comparison with 0.3% dietary NaCl, 2% NaCl increased SBP in both WT ( $P=0.02$ ) and (hAT-rpR) rats ( $P=0.004$ ). When SBP at 12 months was analysed separately, (hAT-rpR) rats receiving 2% dietary NaCl had higher SBP than WT rats receiving either 0.3% ( $P=0.0001$ ) or 2% dietary NaCl ( $P=0.01$ ), and WT rats receiving 2% dietary NaCl had higher SBP than WT rats receiving 0.3% NaCl ( $P=0.02$ ).

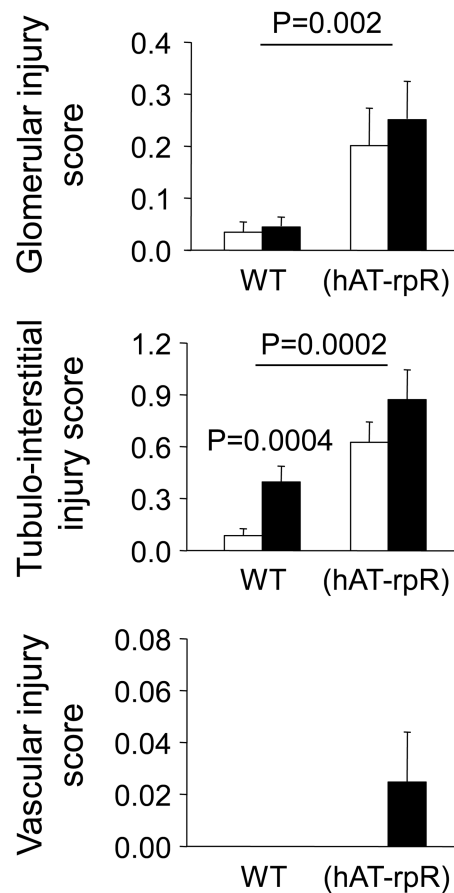
\* $P<0.05$ , † $P<0.01$  for comparison of indicated groups at 12 months of age.



**Figure 2.**

Effect of 0.3% (circles) and 2% (squares) dietary NaCl on plasma prorenin, renin concentration (PRC), and renin activity (PRA) in male wildtype (WT, open symbols) and (hAT-rpR) rats (closed symbols). Data shown as means  $\pm$  SEM,  $n=7-8$ ; where error bars are not shown they are contained within the symbol. For prorenin, 2-way ANOVA for 6-12 months of age showed significant effects of genotype ( $P<0.0001$ ) and dietary NaCl ( $P=0.0003$ ), without interaction between genotype and dietary NaCl, and 2% NaCl reduced prorenin in both WT ( $P=0.01$ ) and (hAT-rpR) rats ( $P=0.0002$ ). When prorenin levels at 12 months were analysed separately, (hAT-rpR) rats had higher prorenin than WT rats for either diet ( $P<0.0001$ ), and 2% dietary NaCl reduced prorenin levels for both (hAT-rpR) ( $P=0.001$ ) and WT rats ( $P=0.04$ ). For PRC, 2-way ANOVA for 6-12 months of age showed significant effects of genotype ( $P<0.0001$ ) and dietary NaCl ( $P=0.003$ ), without interaction between genotype and dietary NaCl, and 2% NaCl reduced PRC in WT ( $P=0.005$ ) but not in (hAT-rpR) rats ( $P=0.06$ ). When PRC at 12 months was analysed separately, (hAT-rpR) rats receiving either 0.3% ( $P=0.0002$ ) or 2% dietary NaCl ( $P=0.001$ ) had higher PRC than WT rats receiving 2% NaCl, and (hAT-rpR) rats receiving 0.3% NaCl had higher PRC than WT rats receiving 0.3% NaCl ( $P=0.03$ ). However, the effect of 2% NaCl on PRC was not statistically significant for either (hAT-rpR) or WT rats at 12 months of age. For PRA, 2-way ANOVA for 6-12 months of age showed significant effects of genotype ( $P<0.0001$ ) and dietary NaCl ( $P<0.0001$ ), without interaction between genotype and dietary NaCl, and 2% NaCl reduced prorenin in both WT ( $P<0.0001$ ) and (hAT-rpR) rats ( $P=0.0003$ ). When PRA at 12 months was analysed separately, (hAT-rpR) rats receiving 0.3% dietary NaCl had higher PRA than WT rats receiving either 0.3% ( $P=0.046$ ) or 2% dietary NaCl ( $P=0.0002$ ), whereas PRA of (hAT-rpR) rats receiving 2% NaCl was not different from that of WT rats receiving either diet. Increased dietary NaCl reduced PRA in both (hAT-rpR) ( $P=0.01$ ) and

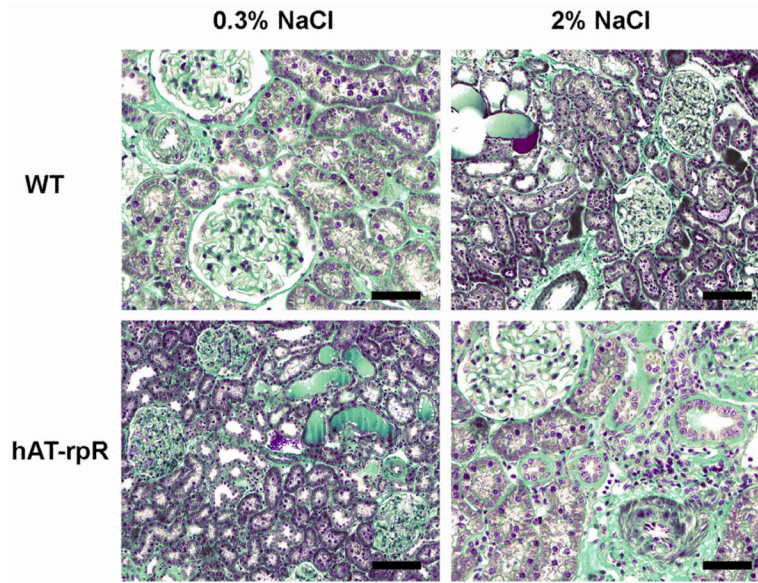
WT rats ( $P=0.047$ ) at 12 months of age.  $*P<0.05$ ,  $†P<0.01$  for comparison of indicated groups at 12 months of age.



**Figure 3.**

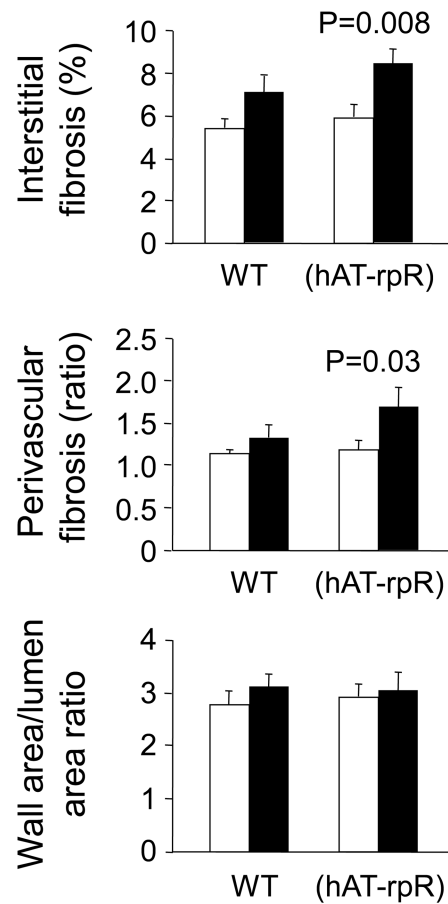
Effect of 0.3% NaCl diet (open columns) and 2% NaCl diet (closed columns) on renal glomerular, tubulo-interstitial, and vascular injury scores in male wildtype (WT) and (hAT-rpR) rats at 12 months of age. Data shown as means  $\pm$  SEM,  $n = 7-8$ . For glomerular injury score, 2-way ANOVA showed a significant effect of genotype ( $P=0.002$ ), but not of dietary NaCl. For tubulo-interstitial injury score, 2-way ANOVA showed a significant effect of both genotype ( $P=0.0002$ ) and dietary NaCl ( $P=0.02$ ); the effect of dietary NaCl was significant for WT rats ( $P=0.0004$ ), but not for (hAT-rpR) rats, when analyzed separately. The vascular injury score was zero for WT rats on either diet and for (hAT-rpR) rats on a 0.3% NaCl diet, and 2-way ANOVA showed no effect of either genotype or dietary NaCl.





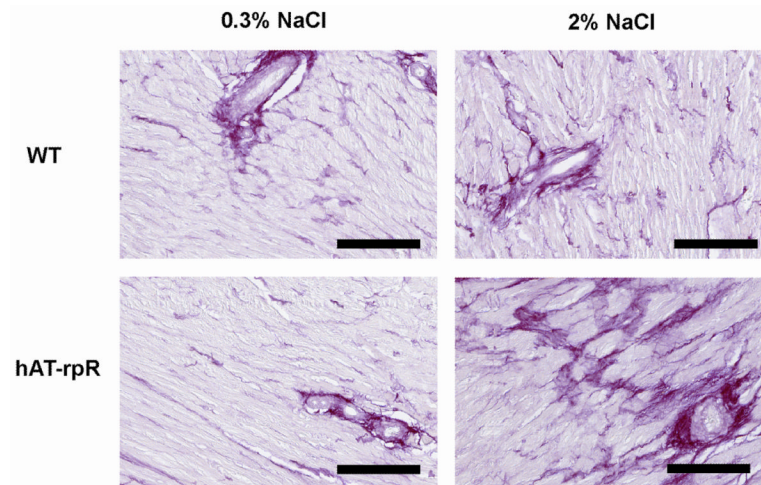
**Figure 4.**

Representative kidney sections of wildtype (WT) and transgenic (hAT-rpR) rats administered either 0.3% or 2% dietary NaCl from 3 to 12 months of age. At 12 months of age, (hAT-rpR) rats receiving on 0.3% dietary NaCl showed glomerulosclerosis, tubulo-interstitial inflammation and fibrosis, tubular atrophy and casts, in comparison with WT rats. In comparison with 0.3% dietary NaCl, 2% dietary NaCl had no affect on glomerular or tubulo-interstitial injury score of (hAT-rpR) rats, whereas WT rats showed increased tubulo-interstitial, but not glomerular, injury score in response to 2% dietary NaCl. Masson's trichrome stain; bar = 50 $\mu$ m.



**Figure 5.**

Effect of 0.3% NaCl diet (open columns) and 2% NaCl diet (closed columns) on myocardial interstitial and perivascular fibrosis, and myocardial vessel wall/lumen ratio in male wildtype (WT) and (hAT-rpR) rats at 12 months of age. Data shown as means  $\pm$  SEM,  $n = 7-8$ . For interstitial fibrosis, 2-way ANOVA showed a significant effect of dietary NaCl ( $P=0.003$ ), but not of genotype; the effect of dietary NaCl was significant for (hAT-rpR) rats ( $P=0.008$ ), but not for WT rats, when analyzed separately. For perivascular fibrosis, 2-way ANOVA showed a significant effect of dietary NaCl ( $P=0.03$ ), but not of genotype; the effect of dietary NaCl was significant for (hAT-rpR) rats ( $P=0.03$ ), but not for WT rats, when analyzed separately. For vascular wall/lumen ratio, 2-way ANOVA showed no significant effect of either genotype or dietary NaCl.



**Figure 6.**

Representative heart sections of wild type (WT) and transgenic (hAT-rpR) rats administered either 0.3% or 2% dietary NaCl from 3 to 12 months of age. At 12 months of age, there was no difference in interstitial or perivascular fibrosis, or myocardial vessel wall/lumen ratio between WT and (hAT-rpR) rats administered 0.3% dietary NaCl. In comparison with 0.3% dietary NaCl, 2% dietary NaCl did not modify myocardial interstitial or perivascular fibrosis in WT rats. However, 2% dietary NaCl increased myocardial interstitial and perivascular fibrosis in (hAT-rpR) rats. Dietary NaCl did not influence myocardial vessel wall/lumen ratio in either WT or (hAT-rpR) rats. Sirius red staining collagen; bar = 200 $\mu$ m.

Table 1

Effect of 0.3% and 2% dietary NaCl on left ventricular and kidney weight, plasma angiotensin I and II and angiotensinogen, and kidney renin in male wild type and (hAT-rpR) rats.

Parameter	Wildtype		(hAT-rpR)		Statistical significance		
	0.3% NaCl	2% NaCl	0.3% NaCl	2% NaCl	Genotype	NaCl	Interaction
LVW (g)	0.71 ± 0.01	0.80 ± 0.03 <sup>*</sup>	0.84 ± 0.02 <sup>§</sup>	0.91 ± 0.03 <sup>*,§</sup>	P<0.0001	P=0.003	NS
LVW/BW ratio (mg/g)	1.78 ± 0.03	1.97 ± 0.03 <sup>*</sup>	2.06 ± 0.04 <sup>§</sup>	2.36 ± 0.08 <sup>*,§</sup>	P<0.0001	P<0.0001	NS
KW (g)	1.33 ± 0.04	1.45 ± 0.08	1.39 ± 0.02	1.44 ± 0.08	NS	NS	NS
KW/BW ratio (mg/g)	3.33 ± 0.05	3.58 ± 0.15	3.45 ± 0.08	3.76 ± 0.19	NS	P=0.04	NS
Plasma Ang I (fmol/mL)	58.2 ± 8.4	20.7 ± 4.7 <sup>‡</sup>	35.6 ± 8.3 <sup>‡</sup>	30.5 ± 7.2	NS	P=0.008	P=0.04
Plasma Ang II (fmol/mL)	17.4 ± 1.8	7.7 ± 1.6 <sup>‡</sup>	15.5 ± 1.8	12.3 ± 1.7	NS	P=0.001	NS
Plasma angiotensinogen (ng Ang I/mL)	999 ± 88	993 ± 95	832 ± 58	729 ± 39 <sup>‡</sup>	P=0.005	NS	NS
Renal renin (µg Ang I/mg protein per h)	2.59 ± 0.74	0.58 ± 0.23 <sup>‡</sup>	0.53 ± 0.08 <sup>§</sup>	0.44 ± 0.08	P=0.01	P=0.002	P=0.01
Aortic wall area (mm <sup>3</sup> )	0.64 ± 0.01	0.66 ± 0.01	0.76 ± 0.03 <sup>§</sup>	0.91 ± 0.04 <sup>*,§</sup>	P<0.0001	P=0.003	P=0.02
Aortic wall thickness (µm)	110 ± 2	111 ± 2	127 ± 3 <sup>§</sup>	145 ± 5 <sup>*,§</sup>	P<0.0001	P=0.004	P=0.01
Aortic cross-sectional area (mm <sup>3</sup> )	2.34 ± 0.10	2.48 ± 0.09	2.61 ± 0.07	2.86 ± 0.12 <sup>§</sup>	P=0.003	NS	NS

Data shown as means ± SEM, n=7-8. Ang I, angiotensin I, Ang II, angiotensin II, BW, body weight; LVW, left ventricular weight; KW, kidney weight. The kidney renin data for WT and (hAT-rpR) rats receiving 2% dietary NaCl were reported previously [7]. Data were analyzed by 2-way ANOVA.

<sup>\*</sup> P<0.05,

<sup>‡</sup> P<0.01, comparison of 2% NaCl with 0.3% NaCl for each genotype after adjustment for multiple comparisons;

<sup>§</sup> P<0.05,

<sup>§</sup> P<0.01, comparison of (hAT-rpR) with WT rats receiving same diet, after adjustment for multiple comparisons